(UNEXECUTED DISCUSSION DRAFT)

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent application of: Leadlay *et al.*,

Serial No:	09/214,453	Examiner:	Kathleen M. Kerr
Filed:	5 January 1999	Art Unit:	1652
For:	Polyketides and their synthesis	Docket No:	

DECLARATION UNDER 37 CFR '132

Assistant Commissioner for Patents
Washington, DC 20231

SIR:

I, Jeremy Randall Knowles, hereby declare:

1) I am currently the Amory Houghton Professor of Chemistry and Biochemistry in the Faculty of Arts and Sciences at Harvard University. My professional career in research and teaching began at the University of Oxford in the early 1960s, and I joined the faculty of Harvard University in 1974. My full CV is attached as annex 1.

2) I have led a major research effort and lectured extensively on mechanistic enzymology for nearly forty years, and I have published more than 250 papers and reviews in this field. I have worked on mechanism of enzyme action, on the relationship between the structure and function of enzymes, on the evolution of enzyme function, on mutagenesis and selection, and on stereochemical aspects of enzyme catalysis. My research has ranged from mechanistic studies of the reactions of primary and secondary metabolism, to the manipulation of micro-organisms and their proteins using the techniques of molecular biology.

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3) I have read US5962290 ("Khosla '290") and the specification of the above-identified application, initially published as WO98/01546.

4) I am familiar with the state of the art at the priority date of the above-identified application, namely 5 July 1996. Specifically, I have reviewed the documents in the list of references attached as annex 2ed hereto.

5) I am commenting on the teaching of Khosla '290 and what this can reasonably be said to place in the public's possession.

6) The genetics and enzymology of polyketide biosynthesis has been an area of particularly active research since the mid-1980s. It is now well-appreciated that the biosynthesis of polyketide compounds involves successive condensation reactions of acyl units (as acyl-CoA esters) that are joined to form a growing β-ketoacyl chain. Single units may undergo modification (for example, to different levels of reduction) before further chain extension by condensation with another acyl-CoA unit. The overall process is under the control of a polyketide synthase (a "PKS"), which is a multi-functional protein or a complex of mono-functional proteins that contains the necessary enzymic activities for polyketide biosynthesis and controls the folding, cyclisation, and ultimate length of the acyl chain.

7) In bacteria, two distinct types of PKS were known at the effective date (5th July 1996). Type II PKSs are responsible for the biosynthesis of the *aromatic* polyketides, such as actinorhodin, frenolicin, tetracycline, and daunorubicin. In contrast, Type I PKSs are responsible for the biosynthesis of more complex (and often reduced) polyketides, such as erythromycin, rapamycin, tylosin, avermectin and amphotericin. The two types of PKS have very different structures, and their biosynthetic targets are assembled in quite different ways.

8) The first Type II PKS gene cluster to be identified, that for actinorhodin from *Streptomyces coelicolor*, was isolated and heterologously expressed in 1984 (Malpartida & Hopwood, *Nature*, 1984, **309**, 462-464). By 1995, the gene sequences for at least 13 Type II PKSs from different *Streptomyces* sp cies

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had been identified and characterised, establishing the pattern and basic composition of these Type II

PKSs (Hutchinson & Fujii, *Annu. Rev. Microbiol.*, 1995, **49**, 201-238). Type II PKSs were found to

consist of several different, largely mono-functional proteins, and to contain only a single copy of the

gene for each of the enzyme activities required for chain extension (Hopwood & Sherman, *Annu. Rev.*

Genet. 1990, **24**, 37-66; Katz & Donadio, *Annu. Rev. Microbiol.* 1993, **47**, 875-912; Shen &

Hutchinson, *Science,* 1993, **262**, 1535-1540). The principal mechanistic challenges faced by Type II

PKSs are therefore to control the number of times chain extension occurs before cyclisation and

aromatisation, and to define the folding of the poly-β-ketone intermediates into the correct

conformation for cyclisation. The essential enzymatic components required for the synthesis of a

polyketide chain by a Type II PKS had been shown to be the β-ketoacyl synthase (KS), an acyl carrier

protein (ACP), and a specialised protein, originally designated chain-length-determining-factor, or

'CLF' [since it was then thought to determine the final chain length of the nascent polyketide

(McDaniel *et al.*, *Science*, 1993, **262**, 1546-1550)], but now termed KS. Together, these components

have been termed the minimal PKS (McDaniel *et al.*, *Science*, 1993, **262**, 1546-1550; McDaniel *et al.*,

Proc. Natl. Acad. Sci. U.S.A. 1994, **91**, 11542-11546). The individual enzymes are not covalently

linked to each other, and were thought to associate into some form of functional complex (Shen &

Hutchinson, *Science*, 1993, **262**, 1535-1540), possibly a heterodimer of KS and KS (although little

further information on the actual structure of Type II PKSs had been reported by the effective date in

mid-1996). Since there is only one KS and one ACP component in a Type II PKS, it was concluded

that these enzymes must operate in every cycle of the overall process of polyketide chain assembly.

These enzymes must therefore have a broad range of substrate specificity in terms of the polyketide

chain length that they can handle. In this class of PKS there is a monotony in the chemical nature of

the chain-extension reactions, since the extender is invariably malonyl-ACP and the second substrate

(attached to the KS is almost always a linear poly--oxoacyl chain. The identity of the presumed

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acyltransferase that loads malonyl units from malonyl-CoA onto the ACP was not known at the effective date, and is still a matter of debate.

9) Additional catalytic activities that depend on the particular biosynthetic target are considered to be part of the Type II PKS complex, and include cyclases and aromatases responsible for the conversion of the unstable poly-β-ketone chain to an aromatic product, as well as acyltransferases (usually involved where non-standard starter units are used), and (sometimes) ketoreductases. It remains unclear whether the ketoreductases act on the growing chain or on the full-length chain. The difficulties in assigning a precise biochemical function to each of the component enzymes are exemplified by the fact that the 'CLF' has since been shown to be a decarboxylase responsible for decarboxylating malonyl-CoA (or other) primers for loading onto the ACP (Bisang *et al.*, *Nature*, 1999, **401**, 502-505). This protein is thus now more commonly referred to as KSβ. Furthermore, the aromatase/cyclase components have been shown to exert a direct influence on polyketide chain length (Petkovic *et al.*, *J. Biol. Chem.*, 1999, **274**, 32829-32834), further strengthening the view that these enzymes have a role in the structural and functional integrity of the Type II PKS complex.

10) Novel aromatic polyketides had been produced simply by deletion of genes encoding the mono-functional enzyme components of native Type II PKSs, often using random mutagenesis to generate 'blocked mutant' strains. Many of these blocked mutants helped to determine the sequence of biosynthetic steps leading to production of the final product(s). However, such products were not considered to be 'hybrid,' since this term describes compounds resulting from the direct transfer of cloned genes isolated from one antibiotic producer into a second recipient strain, as was used to produce the compound mederrhodin A (Hopwood *et al.*, *Nature*, 1985, **314**, 642-644; Omura *et al.*, *Antimicrob. Agents and Chemother.*, 1986, **29**, 13-19). Other examples of novel polyketides produced by a 'hybrid' PKS (defined hereinafter as a PKS expressed from genes derived from more than one PKS gene cluster) were provided by Bartel *et al.* (*J. Bacteriol.*, 1990, **172**, 4816-4826), and by Strohl *et al.* (in *Genetics and Molecular Biology of Industrial Microorganisms*, 1989, Ed., Hershberger,

Queener, & Hegeman, p68-84), who produced a range of novel aromatic polyketides by transforming various *S. galilaeus* strains with genes encoding components of the actinorhodin Type II PKS. However, as is the case in the majority of such experiments, the novel polyketides were not processed through to the end-product anthraquinones. Bartel and coworkers did not have access to precise details of the gene arrangement in the *act* locus, but they nevertheless obtained proof of concept. Other novel aromatic compounds were produced in an analogous manner by deletion or disruption of genes encoding Type II polyketide synthase components (using knowledge of the DNA sequence and organization of these components) and their replacement with homologues from other Type II PKS gene clusters (reviewed in Katz & Donadio, *Annu. Rev. Microbiol.*, 1993, **47**, 875-912, see page 891 and references therein; Hutchinson & Fujii, *Annu. Rev. Microbiol.*, 1995, **49**, 201-238; McDaniel *et al., Science*, 1993, **262**, 1546-1550; McDaniel *et al., J. Am. Chem. Soc.*, 1993, **115**, 11671-11675; McDaniel *et al., Proc. Natl. Acad. Sci.* U.S.A. 1994 **91** 11542-11546; McDaniel *et al., J. Am. Chem. Soc.*, 1995, **117**, 6805-6809; McDaniel *et al., Nature*, 1995, **375**, 549-554; Fu *et al., Biochemistry*, 1994, **33**, 9321-9326; Khosla *et al., Mol. Microbiol.*, 1992, **6**, 3237-3249; Khosla *et al., J. Bacteriol.*, 1993, **175**, 2197-2204). The creation of such novel aromatic polyketides was facilitated by the development, in the early 1980s, of cloning vectors that allowed the expression of heterologous genes in *Streptomycetes* (for example, Lydiate *et al., Gene*, 1985, **35**, 223-235). One such expression vector/host combination involves the SCP2-derived low copy-number vector pRM5, and the engineered host *Streptomyces coelicolor* CH999, from which almost the entire actinorhodin PKS gene cluster had been removed by standard methods of chromosomal gene deletion (McDaniel *et al., Science*, 1993, **262** 1546-1550; US5962290).

11) Such 'mixing-and-matching' of Type II PKS genes was frequently successful when the KS and KS were from the same natural PKS, and the ACP, cyclases, reductases and/or aromatases were obtained from different natural PKS gene clusters. However, these experiments were almost uniformly unsuccessful in producing new products when the KS and KS (i.e., the principal chain-building

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enzymes) were obtained from different natural PKS gene clusters (see Hutchinson & Fujii, *Annu. Rev. Microbiol.*, 1995, **49**, 201-238; McDaniel *et al.*, *J. Am. Chem. Soc.*, 1993, 115, 11671-11675; Kim *et al.*, *J. Bacteriol.*, 1992, **176**, 1801-1804; Sherman *et al.*, *J. Bacteriol.*, 1992, **174**, 6184-6190).

12) In contrast to the work described above on Type II PKSs, the first Type I PKS gene cluster was not isolated and characterised until the early 1990s, when the sequence of the genes for 6-deoxyerythronolide B synthase (DEBS), which is the PKS responsible for the biosynthesis of the erythromycin macrolactone, was reported (Cortes *et al.*, *Nature*, 1990, **348**, 176-178; Donadio *et al.*, *Science*, 1991, **252**, 675-679; Bevitt *et al.*, *Eur. J. Biochem.*, 1992, **204**, 39-49). Surprisingly, this first example of a Type I PKS was found to be totally different in structure from the Type II PKSs. The enzyme activities were found not to be physically separate, and were not used many times during chain assembly, as in a Type II PKS. Instead, the enzymes were elements of giant multi-functional proteins, each of which contained multiple versions of the various enzyme activities required, with a separate domain catalysing each individual step in the overall chain assembly process. The domain sequence was found to follow an ordered, repeating, pattern in which the domains are organised into 'modules', each of which carries the domains required for one complete chain extension operation. The length of the resulting polyketide chain was found to be a consequence of the number of modules, each module being responsible for one cycle of chain extension. Every module contained at least three 'core' activities [β –ketoacyl synthase (KS), acyl transferase (AT), and acyl carrier protein (ACP)]. Additionally, each module contained reductive activities [ketoreductase (KR), dehydratase (DH), and enoylreductase (ER)], according to the degree of reduction required in each cycle of chain extension. Initially, the first module was characterised as containing an AT–ACP didomain responsible for priming the PKS with the starter acyl residue (Donadio *et al.*, *Science*, 1991, **252**, 675-679; Aparicio *et al.*, *J. Biol. Chem.*, 1994, **269**, 8524-8528). However, the application in suit (WO98/01546) established that this didomain represented a specialised and functionally autonomous 'loading module' responsible for selection of a primary precursor that serves as the starter acyl unit,

which is then passed to the next module to initiate the first chain extension cycle. It was believed that the growing polyketide chain remains covalently attached to the enzyme *via* a phosphopantetheinyl arm attached to the ACP, and is passed from module to module until it has been processed by the last one, when it is finally released from the PKS. Chain release is achieved by a dedicated thioesterase (TE) attached to the C-terminus of the final chain extension module.

13) The erythronolide sythase (DEBS) remained the only complete Type I PKS for which gene sequence was available, although fragmentary information was published on, for example, the closely-related oleandolide PKS (Swan *et al.*, *Mol. Gen. Genet.*, 1994, **242**, 358-362), and the avermectin PKS (MacNeil *et al.*, *Gene*, 1992, **115**, 119-125; MacNeil *et al.*, in *Industrial Microorganisms: Basic and Applied Molecular Genetics,* 1993, Ed. Baltz, Hegeman, & Skatrud, p245-256), until the publication in August 1995 of the rapamycin PKS gene sequence (Schwecke *et al.*, *Proc. Natl. Acad. Sci., USA,* 1995, **92**, 7839-7843). This sequence revealed significant differences from the erythronolide PKS, particularly in respect of its overall structural characteristics. Thus first, whereas in the erythronolide PKS there is a one-to-one correspondence between active domains present and the final polyketide product, this was found not always to be the case in the rapamycin PKS. The latter enzyme appears to have potentially active KR and DH domains in modules 3 and 6, which are not required. Module 3 also contains a potentially active but seemingly functionally redundant ER domain. Second, the loading module, rather than comprising simply the AT and ACP domains as in the erythronolide PKS, was found to consist of an adenylate-forming domain (termed CoA-ligase) and a ER domain. Third, whereas the largest multifunctional polypeptide in the erythronolide PKS contains a loading module and two extension modules, the longest polypeptide of the rapamycin PKS is a gigantic protein comprising six extension modules. Fourth, the polyketide chain is not released from the final module of the rapamycin PKS by a C-terminal thioesterase (TE), but appears to be passed to an enzyme (dubbed pipecolate-incorporating enzyme: PIE) that resembles non-ribosomal peptide synthetase

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modules in its sequence and that inserts an amino acid prior to the cyclisation of the macrocycle by an as yet unknown mechanism.

14) It had been shown that when the erythronolide synthase DEBS was purified from extracts of the producing organism *Saccharopolyspora erythraea*, the individual multienzymes DEBS1, DEBS2 and DEBS3 could be readily separated from each other by conventional chromatography (Caffrey *et al*, *FEBS Lett.*, 1992, **304**, 225-228). The three different multifunctional polypeptides were proposed to dock together in a transient but specific head-to-tail manner [so that the KS3 domain of DEBS2 interacts with ACP2 of DEBS1, and KS5 of DEBS3 interacts with ACP4 of DEBS2 (Staunton *et al.*, *Nat. Struct. Biol.*, 1996, **3**, 188-192)]. In contrast to these relatively weak interactions, it was known that the active form of a Type I PKS is in fact a homodimeric complex, and that DEBS1, DEBS2 and DEBS3 each contain two tightly-associated identical multifunctional polypeptide chains (Aparicio *et al.*, *J. Biol. Chem.*, 1994, **269**, 8524-8528; Staunton *et al.*, *Nat. Struct. Biol.*, 1996, **3**, 188-192). Two alternative proposals for the quaternary structure of the active complex had been put forward, one involving a helical parallel assembly of homodimers (Staunton *et al.*, *Nat. Struct. Biol.*, 1996, **3**, 188-192), and the other, now disfavored, a set of planar head-to-tail dimers, analogous to the then-prevailing view of the fatty-acid synthase structure (Cortes *et al.*, *Nature*, 1990, **348**, 176; Bevitt *et al.*, *Eur. J. Biochem.*, 1992, **204**, 39-49; Kao *et al.*, Biochemistry, 1996, **35**, 12363-12368). The set of homodimeric multifunctional polypeptides are believed to bind together using docking sequences at the N- and C- termini to ensure their correct orientation. The 'core' of the whole multienzyme, comprising KS, AT and ACP domains within each module, was believed to stabilise the dimeric structure, which in one typical case was shown by analytical ultracentrifugation to be an extremely tight association with a nanomolar dissociation constant (Staunton *et al.*, *Nat. Struct. Biol.*, 1996, **3**, 188-192).

15) In contrast to the situation with Type II PKSs, genetic engineering of Type I PKSs before the effective date was very limited, and was confined to manipulation of the DEBS genes, first through

the random insertion of DNA segments, and then, once the erythronolide PKS had been sequenced, by targeted domain disruption experiments. McAlpine and coworkers had described a recombinant organism that synthesizes small amounts of 2-*nor*erythromycins in addition to erythromycin A (McAlpine *et al., J. Antibiot.,* 1987, **40**, 1115-1122), by transforming a mutant of *Saccharopolyspora erythraea* blocked in erythromycin production with a cosmid library from the oleandomycin producer, *Streptomyces antibioticus.* The lesion in the blocked *Saccharopolyspora* host was later shown to lie within the gene locus encoding the polyketide synthase (Tuan *et al., Gene,* 1990, **90**, 21-29). This result showed that an acetate unit could replace the propionate unit that is normally inserted by the last extension module 6, in both erythromycin and oleandomycin biosynthesis. However, this result was confusing, and it could not (and still cannot) be unambiguously interpreted because the exact nature of the incoming DNA was not known. Indeed, the product formed was wholly unpredictable on the basis of the state of art at that time. Following the sequencing of the PKS gene, it was demonstrated that in-frame deletions in the ketoreductase gene of module 5 of DEBS, and – separately – site-directed changes in the enoylreductase of module 4, led to small amounts of modified erythronolide analogues (Donadio *et al., Science,* 1991, **252**, 675-679; Donadio *et al., Proc. Natl. Acad. Sci. USA,* 1993, **90**, 7119-7123). Selection of sites for deletion was guided by the knowledge that the natural system produced minor shunt metabolites produced by erroneous processing by some of these domains (Donadio *et al., Science,* 1991, **252**, 675-679, see information in reference 21; Martin & Perun, *Biochemistry,* 1968, **7**, 1728). On the other hand, Bevitt *et al. (Biochem. Soc. Transactions,* 1992, **21**, 30S) showed that, after site-directed mutagenesis of the dehydratase active site, the organism failed to produce erythromycin-like products, even though only one residue out of a total of about 30,000 in the DEBS PKS had been altered.

16) It had been shown that the DEBS genes could be individually expressed heterologously and in some cases shown to be functional (Roberts *et al., Eur. J. Biochem.,* 1993, **214**, 305-311; Leadlay *et al., Biochem Soc Transactions,* 1993, **21**, 218-222; Kao *et al., J. Am. Chem. Soc.,* 1994, **116**, 11612-

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11613), and later that a derivative of the pRM5 vector could be used for expression of the entire

unmodified set of DEBS genes in a heterologous organism, *Streptomyces coelicolor* (Kao *et al.*,

Science, 1994, **265**, 509-512). It was also shown that polyketides of smaller ring size could be

produced by engineering the DEBS genes so as to reposition the terminal thioesterase (TE) domain.

The first result was disclosed by Leadlay and co-workers, who fused the gene encoding the TE

domain to the C-terminus of the ACP of module 2 in DEBS1. This construct led to the production of

a 6-membered triketide lactone (P.F. Leadlay *et al.*, *Abstracts of Society for General Microbiology*

meeting, Warwick, January 1994; see also the reference (p 378) to the work of Leadlay *et al.* in

Hutchinson, *Biotechnology*, 1994, **12**, 375-380; Wiesmann *et al.*, *Abstracts of International*

Symposium on Biology of Actinomycetes, Moscow, July 1994, 154; Brown *et al.*, *J. Chem. Soc. Chem.*

Commun., 1995, 1517-8; Cortes *et al*, *Science*, 1995, **268**, 1487-1489). This work was later

supplemented by *in vitro* studies showing that the TE of DEBS has a broad substrate specificity with

respect to chain length (Aggarwal *et al.*, *J. Chem. Soc. Chem. Commun.*, 1995, 1519-1520). The first

result was subsequently replicated by Kao *et al.* (*J. Am. Chem. Soc.*, 1995, **117**, 9105-9106), replicated

in vitro by Wiesmann *et al.* (*Chem. Biol.*, 1995, **2**, 583-589), and repeated for different ring sizes (Kao

et al., *J. Am. Chem. Soc.*, 1996, **118**, 9184-9185; Kao *et al.*, *J. Am. Chem. Soc.*, 1997, **119**, 11339-

11340). Khosla *et al.* classify such a PKS construct as an in-frame deletion mutant (US5962290, see

example 8).

17) On the basis of the foregoing, therefore, at the priority date of WO98/01546, there was no prior art

with respect to the production of functional hybrid Type I PKSs. Indeed, production of new

metabolites even through targeted homologous manipulation of Type I PKS genes was recognised to

"have been largely unsuccessful" (Hutchinson & Fujii, *Annu. Rev. Microbiol.*, 1995, **49**, 201-238).

18) Khosla *et al.* (in US 5,962,290: Khosla '290) are principally concerned with the production of a novel

host-vector system for the expression of heterologous genes (and in particular, PKS genes) in a host

from which the native PKS gene cluster has been almost entirely deleted using standard methods of

chromosomal gene deletion. Thus, *Streptomyces coelicolor* strain CH999 was constructed by deletion of almost the entire Type II PKS gene cluster for the aromatic polyketide actinorhodin. An SCP2-derived low copy-number plasmid, pRM5, acts as a shuttle vector for the transfer of Type II PKS genes into the CH999 host strain. Plasmid pRM5 contains actinorhodin PKS components under the control of the native (*S. coelicolor*-derived) promotor *actI/actIII* and its cognate activator *actII*-ORF4 (see McDaniel *et al., Science,* 1993, **262,** 1546-1550). Replacement of the various *act* genes in pRM5 with Type II PKS genes from other sources followed by transformation of CH999 with the new plasmid vector results in the synthesis of hybrid Type II PKSs which in turn produce novel aromatic polyketides. However, with respect to production of hybrid Type II PKSs, Khosla '290 is anticipated by, e.g., Bartel *et al. (J. Bacteriol.,* 1990, **172,** 4816-4826), and by Strohl *et al.* (in *Genetics and Molecular Biology of Industrial Microorganisms,* 1989, Ed., Hershberger, Queener, & Hegeman p68-84). With respect to Type I PKSs, Khosla '290 discloses the heterologous expression of the genes for the (wild-type) erythronolide PKS (DEBS) in *S. coelicolor* CH999, resulting in the biosynthesis of the natural erythromycin intermediate 6-deoxyerythronolide B in transformed cells. This was achieved by insertion of all three DEBS genes (eryAI, eryAII, and eryAIII) into a pRM5-derived plasmid (obtained by recombination in *E. coli* cells) to produce the shuttle vector, pCK7. This recombination method for the assembly of polyketide genes in heterologous hosts suffers from the severe drawback that uncontrolled and random recombination events are likely in the repetitive DNA that encodes modular PKS multienzymes. Indeed, the methodology disclosed in Khosla '290 has not been used in any published experiments since, whether for native Type I PKS enzymes, or for hybrids of Type I PKSs. Finally, Khosla '290 describes the production of polyketides of smaller ring size by engineering the DEBS genes to reposition the terminal thioesterase (TE) domain to provide truncated products. In this aspect, Khosla '290 is anticipated by the work of Leadlay and co-workers (P.F. Leadlay *et al., Abstracts of Society for General Microbiology meeting,* Warwick, January 1994; see also the reference (p 378) to the work of Leadlay *et al.* in Hutchinson, *Biotechnology,* 1994, **12,** 375-380; Wiesmann *et*

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al., Abstracts of International Symposium on Biology of Actinomycetes, Moscow, July 1994, 154, see also the reference (p 378) to the prior work of Leadlay *et al.* in Hutchinson, *Biotechnology,* 1994, 12, 375-380). Since these experiments do not involve the insertion of DNA encoding part of a PKS derived from another gene cluster they cannot be described as producing a hybrid PKS. None of the disclosures in Khosla '290 involve use of Type I PKS genes in the production of hybrid Type I PKSs of any form.

19) As noted above, at the priority date of the application in suit (WO98/01546), namely 5th July 1996, there had to my knowledge been no disclosure or report of the production of functional hybrid Type I PKSs. Furthermore, based on the prevailing understanding of the structure and function of Type I PKSs at that time, and the relatively low success rates in making much more conservative changes by domain deletion/mutagenesis in the DEBS system, there was little reason to be confident of any success in the approaches described in the application in suit. Specifically, the creation of a hybrid Type I PKS by alteration at the domain level or the module level, necessarily requires a significant alteration of the primary sequence of the gene product. It involves the creation of at least one protein splice site between the existing PKS and the inserted material. As a result, there would be strong grounds to suppose, even assuming that the genetic engineering were successful and a recombinant gene product were expressed, that the hybrid Type I PKS might be non-functional, because:

- The hybrid protein with a significantly altered primary sequence may not fold properly;

- Even if the individual domains fold properly, distortions may alter the quaternary structure so that co-operating domains can no longer reach each other, or that the association of a pair of multienzymes to form the active homodimeric complex cannot occur;

- Downstream domains may have narrow substrate specificities and consequently not accept the novel structures passed on by an altered domain or module. In the case of the DEBS1 PKS containing a repositioned TE, the thioesterase only has to catalyse the removal of the polyketide product from the protein complex, and considerations of substrate specificity have limited relevance in this case. These

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results cannot therefore be used to support a view of flexibility and promiscuity of each and every enzymatic activity in the PKS;

- The altered domain may simply not recognise the polyketide chain delivered to it by upstream domains as a substrate.

Furthermore, it was not clear at the priority date of the application in suit that different natural Type I PKSs would have sufficiently similar structures and domain organisations to allow the creation of functional hybrids. Indeed, information from the rapamycin PKS gene sequence published in August 1995 pointed to significant differences between the only two complete Type I PKS sequences then available.

5) Type II PKSs are so radically different in structure and mode of operation from Type I PKSs that extrapolation of results from the former system could not be a meaningful guide as to what might be achievable in the latter. It was generally considered that the obvious major differences in gene sequence and overall protein quaternary structure between the two classes of polyketide synthase would require "two entirely different programming strategies" (McDaniel *et al., Science,* 1993, **262,** 1546-1550). Creation of hybrid Type II PKSs merely required expression of a foreign gene for a particular enzyme of a PKS in a suitable host containing the complementary genes for Type II polyketide biosynthesis. Unlike the generation of a hybrid Type I PKS, the design of precise and appropriate protein splice sites is not required for Type II enzymes. As long as the foreign gene is expressed, the foreign enzyme could replace (or be present in addition to) the equivalent native enzyme in the host PKS, and a hybrid PKS protein complex would be created. The chances of the hybrid Type II PKS being functional, at least in part, are relatively high, for the following reasons. First, there is no need to alter the inserted gene, or any host gene, in a way that alters the amino acid sequence of any gene product. Second, neither the foreign gene product nor its partner gene products from the host PKS will be expected to accept radically different substrates because there is very much less diversity in the poly-β-ketone intermediates generated by Type II PKSs. Even so, the success rate in experiments where the chain-building KS and KS components were

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heterologous to one another was found to be low (Hutchinson & Fujii, *Annu. Rev. Microbiol.*, 1995, **49**, 201-238; Kim *et al.*, *J. Bacteriol.*, 1992, **176**, 1801-1804; Sherman *et al.*, *J. Bacteriol.*, 1992, **174**, 6184-6190). These results teach us that the engineering of PKS hybrids using domains of heterologous chain-building enzymes has a low probability of success *even in the Type II system*. Thus neither this prior art, nor the Khosla '290 application, can properly be used to suggest that heterologous mixing experiments are obvious in the Type I system. Furthermore, the Type II prior art (which forms the basis of Khosla '290) shows that the engineering of hybrid Type II PKS-containing cells usually leads to the production of complex mixtures of polyketides. These mixtures contain a variety of different structures from which only one or a few of the major components were characterised. There is evidently a marked loss of control over the course of polyketide chain synthesis in hybrid Type II PKSs. Such experiments also teach us that enzymes acting later in polyketide production are significantly less effective in handling modified intermediates. A person skilled in the art would readily perceive that all these problems are further compounded by the intrinsic chemical reactivity of poly--oxoacyl chains, which means that some of the products of hybrid PKSs do not arise through the direct action of (for example) a heterologous PKS cyclase or other "tailoring" enzyme, but rather through a disruption of the normal protein:protein interactions in the native PKS complex that leaves the polyketide chain more susceptible to chemical side-reactions. Taken together, this prior art on Type II PKSs suggests the *improbability* that hybrid Type I PKS enzymes (which necessarily also involve extensive protein: protein interactions) would be functional.

6) Although the sequential reaction "programming" of Type I polyketide synthases is more apparent than it is for Type II systems (indeed, the Type II systems remain poorly understood, with the identity of the enzymes responsible for chain initiation, recruitment of extender units, chain length determination and chain release all still in doubt) a person skilled in the art before the effective date would not have considered that productive hybrid Type I systems were obviously accessible by the methods of the present invention, even in light of Khosla '290. Nor would such a person have believed that manipulation -

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particularly of the 'core' of a modular PKS (the KS, the ACP, and the AT domains) - would (without the inventive step of the present application) give rise to active hybrid multienzymes capable of the synthesis of hybrid polyketides.

7) In my opinion, therefore, it was not obvious prior to the priority date of 5 July 1996 that the strategy described in the application in suit would be successful in producing functional hybrid Type I PKSs. The naïve presumption of Khosla '290 is the metaphorical equivalent of imagining that arbitrarily changing the order of operations in an automobile assembly line would produce a product of any kind (never mind a useful or recognisable one).

8) The disclosure of Khosla '290 provided no incentive to investigate the strategy of the application in suit. Khosla '290 does not disclose any examples of Type I/Type I hybrid PKSs. Furthermore, it does not disclose any credible strategy for creating such hybrids.

9) I hereby declare that all the statements made herein of my own knowledge are true and that all statements made on the information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such wilful statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

Date:_____   _____

Professor Jeremy R. Knowles

Patents

US5962290

WO98/01546

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Sir Louis Stuart Exhibitioner, Balliol College, Oxford, 1955-1959
B.A., Final Honour School of Chemistry, 1959 (1st Class) (Thesis:
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Harmsworth Scholar, Merton College, Oxford, 1959-1960
Research Lecturer, Christ Church, Oxford, 1960-1962
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Fellow of Wadham College, Oxford, 1962-1974
Departmental Demonstrator, Dyson Perrins Laboratory, Oxford, 1962-1966
University Lecturer, University of Oxford, 1966-1974
Visiting Professor, Yale University, 1969 (Molecular Biophysics),
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Sloan Visiting Professor, Harvard University, 1973
Professor of Chemistry, Harvard University, 1974-
Amory Houghton Professor of Chemistry and Biochemistry,
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Chairman, Department of Chemistry, Harvard University, 1980-1983
Dean of the Faculty of Arts and Sciences, Harvard University, 1991-2002

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Charmian Medal, Royal Society of Chemistry, 1981
Fellow of the American Academy of Arts and Sciences, 1982
Newton Abraham Visiting Professor, Oxford University, 1983-84
Honorary Fellow, Balliol College, Oxford, 1984
Kenner Prize, 1985
Foreign Associate, National Academy of Sciences, 1988
Member, American Philosophical Society, 1988

Guthikonda Award, 1988
Alfred Bader Award, 1989
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Prelog Medal, 1989
Honorary Fellow, Wadham College, Oxford, 1990
Smissman Medal, 1990
Honorary Fellow, Royal Society of Chemistry (London), 1991
Davy Medal, Royal Society (London), 1991
Repligen Award, 1992
Doctor, *honoris causa*, University of Edinburgh, 1992
Commander of the British Empire (Queen's Birthday Honours), 1993
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Doctor, *honoris causa*, Eidgenössische Technische Hochschule, Zürich, 2001
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NAMED LECTURESHIPS:
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Philips Lectures, Haverford College, Pennsylvania, 1978
Lutz Lecture, University of Virginia, 1980
Byron Riegel Lectures, Northwestern University, Illinois, 1980
Dreyfus Distinguished Lecturer, Vanderbilt University, 1981
Troy C. Daniels Lectures, University of California, San Francisco, 1982
Cassett Lecture, Temple University, Philadelphia, 1982
T. Y. Shen Lectures in Medicinal Chemistry, MIT, Cambridge, 1982
O. Hayaishi Plenary Lecture, 12th International Congress of Biochemistry, Perth,
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Camille and Henry Dreyfus Lecturer, California Institute of Technology, 1983
Keynote Lecturer, Western Central States Biochemistry Conference,
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College of Science Lectures, University of Notre Dame, Indiana, 1985
George Kenner Lecture, University of Liverpool, U.K., 1985
Smith Kline & French Distinguished Lecturer, Pennsylvania, 1985
Ada Doisey Lecturer, University of Illinois, 1985
Kharasch Visiting Professor, University of Chicago, 1985
Calbiochem-Behring Lecturer, University of California, San Diego, 1986
Venable Lecturer, University of North Carolina, Chapel Hill, 1986
Cynthia Chan Memorial Lectures, University of California, Berkeley, 1986
Gerhard Schmidt Memorial Lecture, Weizmann Institute of Science, 1986
Hirschmann Lecture, Oberlin College, Ohio, 1986
Frontiers in Chemical Research Lectures, Texas A&M University, 1987
Romanes Lecture, University of Edinburgh, U.K., 1987
Mildred Cohn Lecture, University of Pennsylvania, 1987
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Sandin Lectures, University of Alberta, 1987
Chambers Memorial Lectures, University of Rochester, 1988
Barton Lecture, University of Oklahoma, 1988
Bernard Axelrod Lecture, Purdue University, 1988
Plenary Lecturer, Protein Structure and Enzyme Catalysis, Mosbach, 1988

Plenary Lecturer, joint meeting NZIC & NZBS, New Zealand, 1988
Guthikonda Memorial Lecture, Columbia University, 1988
Baker Lectures in Chemistry, Cornell University, 1988
W.S. Johnson Symposium in Organic Chemistry, Stanford University, 1988
Keynote Lecturer, UCLA Symposium on Protein and Pharmaceutical Engineering, 1989
Robbins Lecturer, Pomona College, 1989
Stein Memorial Lecturer, Rockefeller University, 1989
John Stauffer Distinguished Lecture, University of Southern California, 1989
Frontiers of Biological Science Lecturer, Case Western Reserve University, 1989
Kolthoff Lecturer, University of Minnesota, 1989
Leermakers Symposium Lecturer, Wesleyan University, 1989
Plenary Lecturer, Prospects in Protein Engineering, Groningen, 1989
Prelog Lecture, ETH, Zurich, 1989
Marker Lecturer, University of Maryland, 1990
Trends in Biochemistry and Molecular Biology, Torremolinos, Spain, 1990
Leslie Hellerman Memorial Lecturer, Johns Hopkins University, 1990
Plenary Lecturer, Frontiers in Enzyme Chemistry, University of Illinois, 1990
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Allergan Lecturer, University of California, Irvine, 1990
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Smissman Lecturer, University of Kansas, 1990
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MESSAGE:

For consideration in connection with February 7, 2003 interview on U.S. Patent Application No. 09/214,453 of Peter F. Leadlay et al.